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Retrovirus infection strongly enhances scrapie infectivity release in cell culture

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Prion diseases are neurodegenerative disorders associated in most cases with the accumulation in the central nervous system of PrP^{Sc} (conformationally altered isoform of cellular prion protein (PrP^{C}); Sc for scrapie), a partially protease-resistant isoform of the PrP^{C}. PrP^{Sc} is thought to be the causative agent of transmissible spongiform encephalopathies. The mechanisms involved in the intercellular transfer of PrP^{Sc} are still enigmatic. Recently, small cellular vesicles of endosomal origin called exosomes have been proposed to contribute to the spread of prions in cell culture models. Retroviruses such as murine leukemia virus (MuLV) or human immunodeficiency virus type 1 (HIV-1) have been shown to assemble and bud into detergent-resistant microdomains and into intracellular compartments such as late endosomes/multivesicular bodies. Here we report that moloney murine leukemia virus (MoMuLV) infection strongly enhances the release of scrapie infectivity in the supernatant of coinfected cells. Under these conditions, we found that PrP^{C}, PrP^{Sc} and scrapie infectivity are recruited by both MuLV virions and exosomes. We propose that retroviruses can be important cofactors involved in the spread of the pathological prion agent.

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Introduction

The cellular prion protein (PrP^{C}) is a GPI-anchored protein expressed in almost all tissues and predominantly in the central nervous system. PrP^{C} is found in detergent-resistant microdomains (DRMs)/rafts and cycles between the cell surface and endosomal compartments (Vey *et al*, 1996; Naslavsky *et al*, 1997; Peters *et al*, 2003; Sunyach *et al*, 2003). The gene encoding PrP^{C}, *Prn-p*, was shown to be essential for the susceptibility to transmissible spongiform encephalopathie (TSEs) in mice (Bueler *et al*, 1993). TSEs are neurodegenerative diseases characterized by the accumulation in the brain of a conformationally altered isoform of PrP^{C} designated PrP^{Sc} (Sc for Scrapie; Prusiner, 1998). PrP^{Sc} differs from its normal isoform by its high content in β-sheet structure, its insolubility in mild detergents and its partial resistance to proteinase K (PK) treatment. The mechanism and the pathway by which the infectious prion spreads from cell to cell are still enigmatic. Indeed, it has been reported that conditioned medium of scrapie-infected GT1 cells is capable of transmitting infectivity to other cells, while in the case of scrapie-infected SMB cells, a direct cell-to-cell contact is required for transmission (Schatzl *et al*, 1997; Kanu *et al*, 2002). Recently, we found that both PrP^{C} and PrP^{Sc} were released into the extracellular medium of scrapie-infected Rov and Mov cells in association with exosomes (Fevrier *et al*, 2004). Retroviruses and exosomes display many similarities with respect to lipid composition, cellular protein content and the site of assembly and release (Pelchen-Matthews *et al*, 2004).

Retrovirus assembly is a multiple step process orchestrated by the viral Gag polyprotein, the genomic RNA and the cellular membrane in which the viral Envelope glycoproteins are anchored (Cimarelli and Darlix, 2002). In addition, several cellular proteins involved in membrane trafficking are required for particle trafficking and budding (Morita and Sundquist, 2004).

Recent data indicated that retrovirus assembly is more complex than previously thought since murine leukemia virus (MuLV) and human immunodeficiency virus type 1 (HIV-1) assembly can take place in DRMs and, like exosomes, in intracellular compartments that display the hallmarks of late endosomes (Raposo *et al*, 2002; Basyuk *et al*, 2003; Pelchen-Matthews *et al*, 2003; Sherer *et al*, 2003). In agreement with this, several reports indicated that the site of virus assembly influences the protein and the lipid composition of the retroviral envelope. Indeed, host proteins from endosomal compartments or DRMs such as GPI-anchored proteins are recruited into nascent viral particles (Ott, 1997; Campbell *et al*, 2001; Raposo *et al*, 2002; Pelchen-Matthews *et al*, 2003) where they retain their biological function and are able to influence virus replication and the cell physiology (Ott, 1997; Campbell *et al*, 2001; Cantin *et al*, 2005). Since prion proteins and assembling retroviral particles seem to colocalize to the same intracellular compartments, this favors the notion that retroviruses could recruit prion proteins during assembly and budding. Such viruses may thus contribute to the release and the spread of prion proteins. Here we report that MoMuLV infection and viral particle production strongly enhance the release of PrP^{C}, PrP^{Sc} and prion infectivity by coinfected cells. In addition, both PrP^{C} and PrP^{Sc} are found associated with

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MoMuLV particles and exosomes. We propose that retroviruses could be cofactors involved in the spreading of the pathological prion agent.

Results

MoMuLV recruits murine PrP^C

Retroviruses such as MoMuLV use DRMs and endosomal compartments as sites for viral particle assembly and budding (Pickl *et al*, 2001; Basyuk *et al*, 2003; Sherer *et al*, 2003). This prompted us to analyze the colocalization of MoMuLV Gag and Env with murine prion protein. We first analyzed the presence of PrP^C in isolated DRMs (see Supplementary Materials and methods) from NIH3T3 cells infected with MoMuLV (NIH3T3i). Results indicate that part of Gag and Env cofractionates with PrP^c and GM1 in DRMs (Figure 1A, lanes 3–5). To determine if viral Gag and Env, and PrP^c cofractionate in intracellular compartments, NIH3T3i cell extracts were fractionated through optiprep gradient as described previously (Kolesnikova *et al*, 2004). This method allows the separation of membrane proteins associated with the plasma membrane and intracellular compartments. Results presented in Figure 1B show that Gag and Env cofractionate with PrP^C at the plasma membrane (lanes 1 and 2) and in intracellular compartments of endosomal/lysosomal origin as indicated by the presence of Lamp1 (lanes 8–11) and Rab11 (lanes 9, 10 and 17, 18). Immunogold labeling experiments (IEM) with anti-Lamp1 antibodies confirm the presence of virions in Lamp1-positive intracellular organelles where Lamp1 is associated with virions (data not shown). The cofractionation of PrP^C with MoMuLV Gag and Env prompted us to further investigate the recruitment of PrP^C by viral particles. To analyze the possible recruitment of PrP^C by MoMuLV, virions were first concentrated and purified through a 6–18% optiprep gradient. Fractions were analyzed by Western blotting using anti-CAp30 and anti-PrP antibodies. Figure 2A shows that PrP^C and MoMuLV CAp30 cofractionate (fractions 13–16) together with the GM1 raft marker and with reverse transcriptase (RT) (data not shown). To determine if PrP^C was physically associated with virions, immunoprecipitation experiments were realized using magnetic beads conjugated with anti-PrP or the HIV-1 anti-CAp24 as a negative control (Supplementary Materials and methods). Results show that MoMuLV virions were specifically immunoprecipitated by the anti-PrP antibodies (Figure 2B). To confirm the association of PrP^C with MoMuLV, IEM were realized (see Supplementary Materials and methods). To this end, ultrathin cryosections of NIH3T3i cells were immunogold labeled with anti-PrP and anti-CAp30 antibodies. Figure 2(c1–c4) reveals that large amounts of MoMuLV virions are present in intracellular compartments morphologically similar to multivesicular

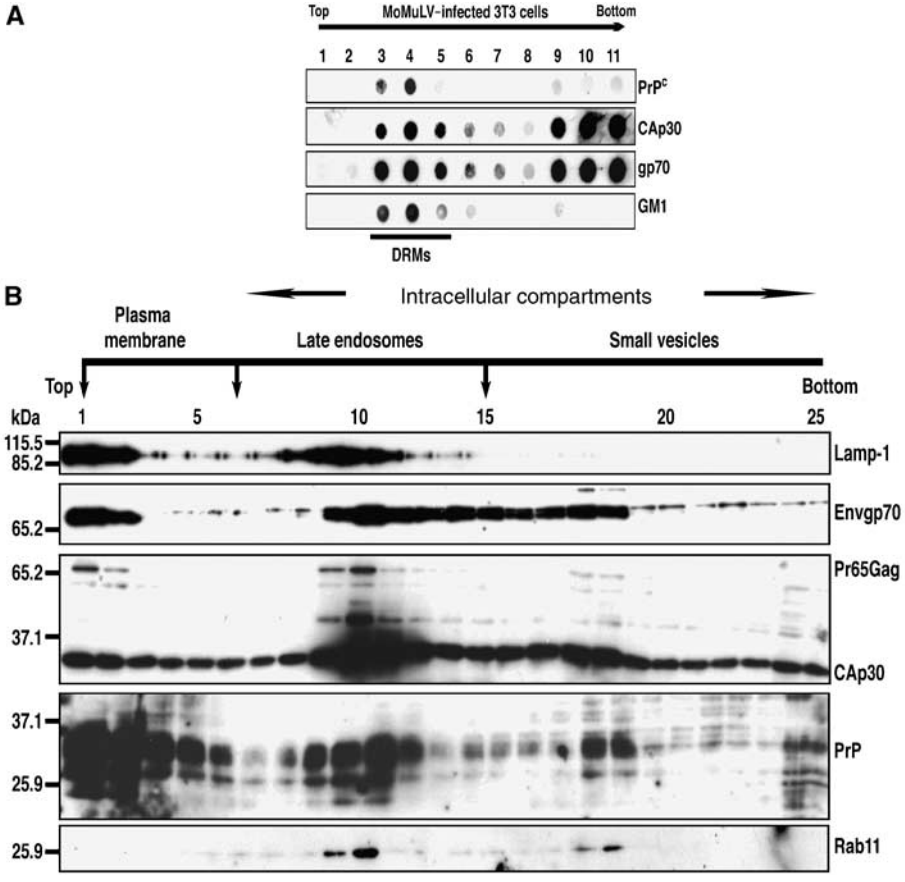

Figure 1 PrP^C cofractionates with MoMuLV Gag and Env. (**A**) DRMs isolation from NIH3T3i cells. Fractions collected from the top of the gradient were analyzed by dot immunoblotting using anti-CAp30, anti-Envgp70 and anti-PrP antibodies. The GM1 raft marker was detected using the BCTx. (**B**) Fractionation of NIH3T3i cell extract by optiprep gradient centrifugation. Fractions were analyzed by immunoblotting using anti-CAp30, anti-Env and anti-PrP antibodies, and profiles were compared with the distribution of cellular marker proteins of the late endosomes/lysosome (Lamp1) compartments and recycling endosomes/small vesicles (Rab11).

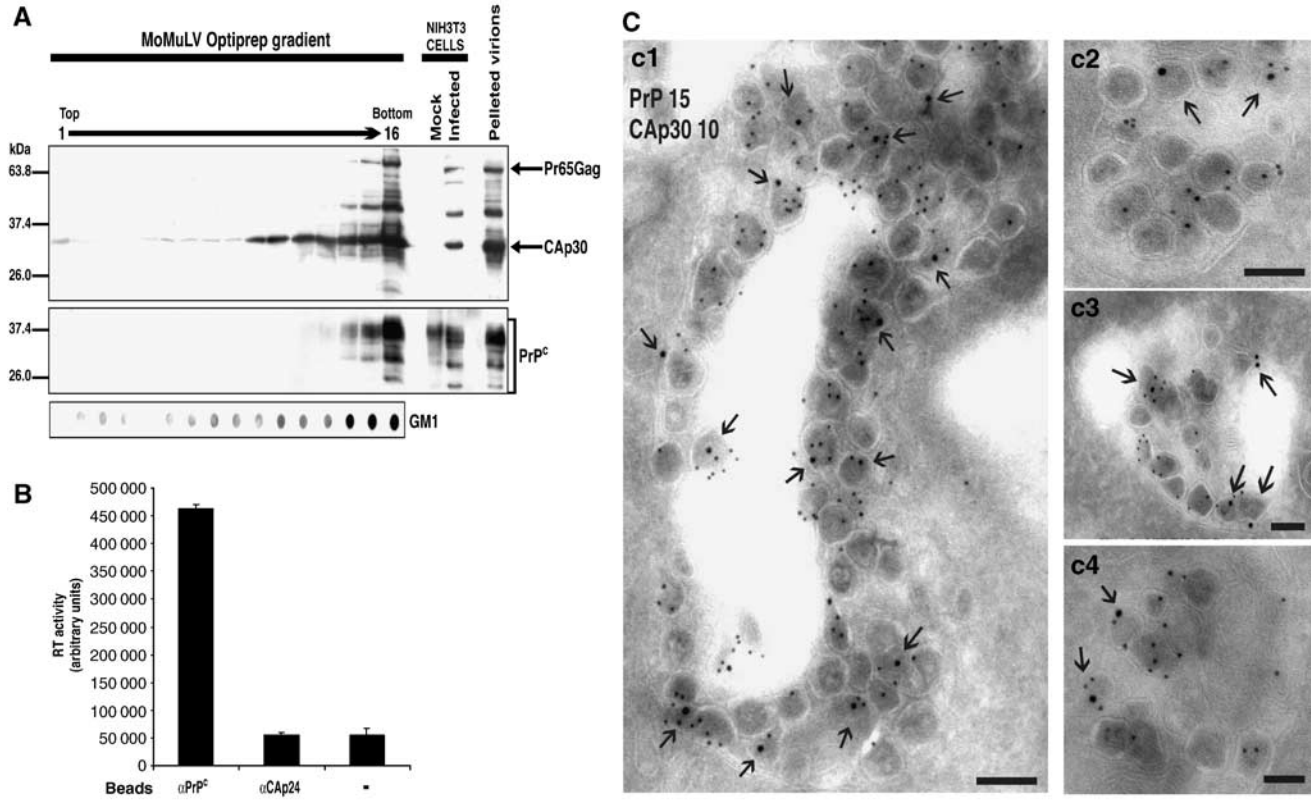

Figure 2 Recruitment of PrP^C by MoMuLV. (**A**) Viral supernatants were recovered and virions were fractionated through a 6–18% optiprep gradient. Fractions were analyzed by Western blotting using anti-CAp30 and anti-PrP antibodies or biotinylated cholera toxin (BCTx) for the GM1 marker. As controls, the mock NIH3T3, the NIH3T3i-infected cell extracts and the 20% sucrose cushion-pelleted virions are shown on the right lanes. (**B**). Immunocapture of MoMuLV virions using anti-PrP antibodies. MoMuLV virions immunoprecipitated with magnetic beads conjugated to anti-PrP antibody or with an anti-HIV-1-CAp24 and beads alone as negative controls. After extensive washing, RT activity was detected on immunocaptured virions. Results are representative of three independent experiments and show that anti-PrP antibodies immunoprecipitate MoMuLV virions. Error bars correspond to means $\pm$ s.d. (**C**) Double immunogold labeling of ultrathin cryosections of NIH3T3i cells using anti-PrP (PAG15) and anti-CAp30 (PAG10) antibodies (c1–c4). Scale bar 100 nm.

bodies (MVBs) and that viral particles consistently display PrP labeling at their surface (see arrows). These data show that PrP^C is recruited by MoMuLV virions. To determine if incorporation of PrP can also take place with other retroviruses, we investigated the recruitment of PrP by a lentivirus such as HIV-1 (Leblanc *et al*, 2004). Our data revealed that human PrP^C is also recruited by HIV-1 (Supplementary Figure 1).

Since PrP remained raft-associated even when the GPI anchor is deleted (PrP-ΔGPI) (Walmsley *et al*, 2003), we wondered if the murine and human PrP-ΔGPI mutant could be recruited into MoMuLV and HIV-1 viral particles. Results indicate that murine and human PrP-ΔGPI are recruited by MoMuLV and HIV-1 viral particles respectively (Supplementary Figure 2).

MoMuLV enhances PrP^C and PrP^{Sc} proteins release

To determine if PrP^{Sc} can be recruited by MoMuLV particles, we used NIH3T3 cells infected by the 22L scrapie prion strain (Vorberg *et al*, 2004b). For this purpose, NIH3T3-N and NIH3T3-22L cells, corresponding to normal and scrapie-infected cells, respectively, were infected with MoMuLV to generate the NIH3T3-N-MoMuLV and NIH3T3-22L-MoMuLV cell lines. Production of MoMuLV virions was assessed by Western blotting using anti-CAp30 and anti-Envgp70 antibodies (Figure 3A, lanes 2 and 4) and by RT activity (data not shown). The presence of PrP^{Sc} was determined by PK treatment followed by Western blotting (Figure 3B, lane 7). No modification of PrP PK resistance was observed in the presence of MoMuLV expression in NIH3T3-22L cells. As expected, PrP^{Sc} was not detected in NIH3T3-N-MoMuLV cells (lane 5). As reported in Figure 1A, Gag and Env cofractionated with PrP^C but also with PrP^{Sc} in DRMs (Figure 3C, lanes 3–5) and soluble fractions (lanes 9–11). These results indicate that PrP^{Sc} cofractionates with assembling viral particles opening the possibility that PrP^{Sc} could be incorporated into MoMuLV virions during the assembly and/or budding process.

To investigate if MoMuLV infection can enhance the extracellular release of prion proteins, the supernatant of NIH3T3-22L cells infected by MoMuLV was harvested and submitted to sequential centrifugations with increasing centrifugation forces (Figure 4A, lanes 5–8). As controls, equal amounts of supernatant of NIH3T3-22L cells (lanes 1–4) and complete DMEM culture medium (lanes 9–12) were used. Western blotting analyses were realized using MoMuLV-specific anti-Envgp70 and anti-CAp30 antibodies, and the result confirmed that only NIH3T3-22L-MoMuLV cells release viral particles in the supernatant with most of them found in the 100 000 *g* pellet (i.e. 100K pellet, see Envgp70 and CAp30/Pr65Gag signals in Figure 4A, lane 8). No viral protein was recovered in the 100K pellet from the control cell supernatants (lanes 4

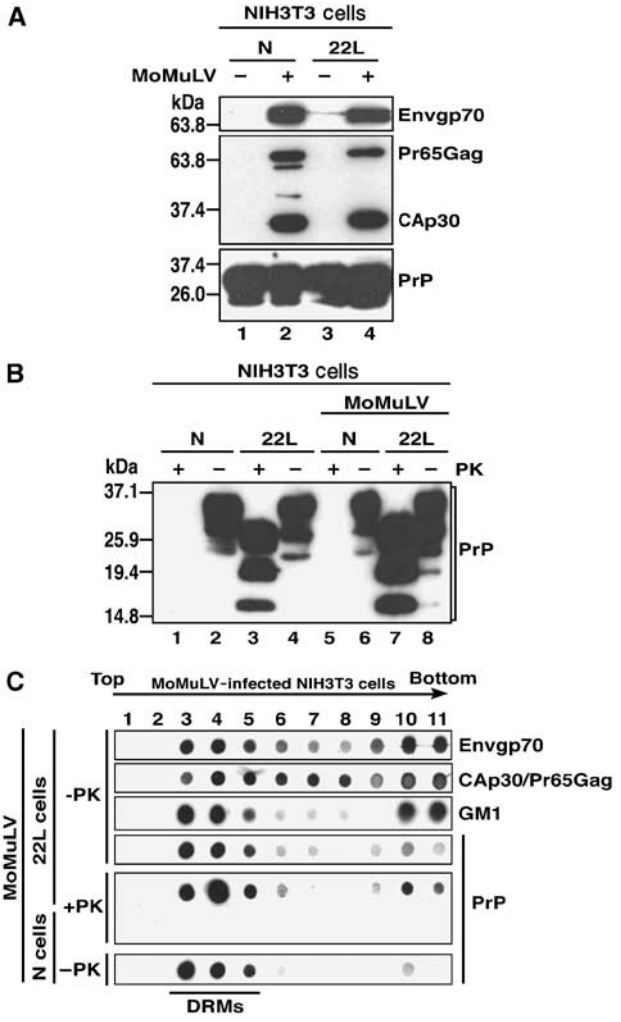

Figure 3 Infection of NIH3T3-N and NIH3T3-22L cells by MoMuLV. (**A**) MoMuLV expression was verified by Western blotting using anti-Envgp70 (top panel), anti-CAp30 (medium panel) and anti-PrP (bottom panel) antibodies. Lane 1: NIH3T3-N; lane 2: NIH3T3-N-MoMuLV; lane 3: NIH3T3-22L; and lane 4: NIH3T3-22L-MoMuLV. (**B**) No modification of PrP PK resistance is induced by MoMuLV infection. Lanes 1–2: NIH3T3-N; lanes 3–4: NIH3T3-22L; lanes 5–6: NIH3T3-N-MoMuLV; lanes 7–8: NIH3T3-22L-MoMuLV; and lanes 1, 3, 5 and 7: PK treated. (**C**) MoMuLV Gag and Env cofractionate with PrP^{C} and PrP^{Sc} in DRMs from NIH3T3-22L-MoMuLV cells. DRMs from NIH3T3-22L-MoMuLV and NIH3T3-N-MoMuLV cells were isolated by equilibrium centrifugation gradient. Fractions were analyzed by dot immunoblotting using the anti-Envgp70, anti-CAp30, anti-PrP antibodies and the BCTx for the GM1 raft marker. For PrP^{Sc} detection, each fraction from NIH3T3-N-MoMuLV (negative control) and NIH3T3-22L-MoMuLV (positive for PrP^{Sc}) was treated by PK. PK-resistant products were analyzed as above by dot immunoblotting. +PK and −PK indicate the presence or the absence of PK treatment.

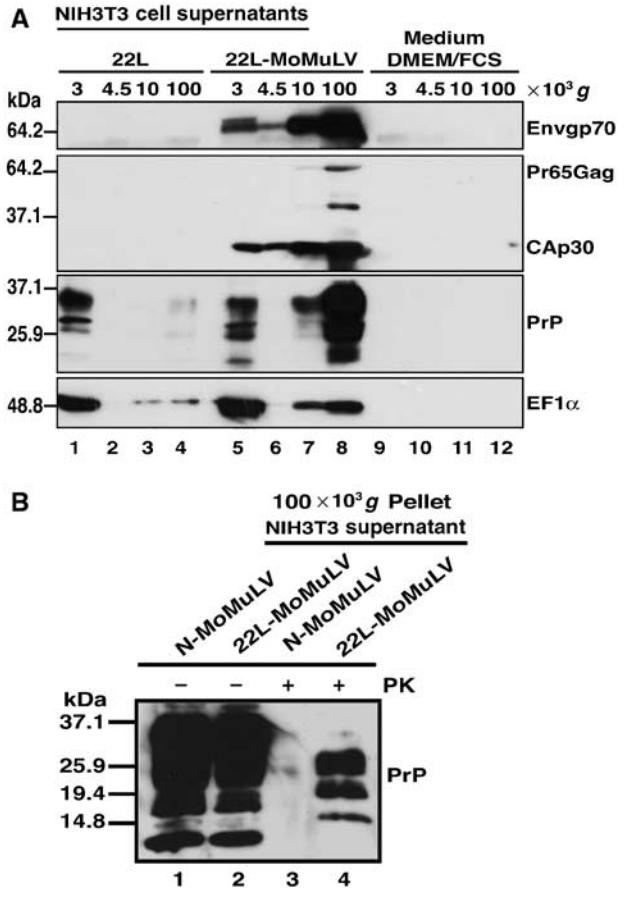

Figure 4 MoMuLV infection strongly enhances prion proteins release. (**A**) Supernatant from NIH3T3-22L (lanes 1–4), NIH3T3-22L-MoMuLV (lanes 5–8) and free complete medium as negative control (lanes 9–12) were submitted to differential centrifugation. Lanes 1, 5 and 9: 3000 *g* for 5 min; lanes 2, 6 and 10: 4500 *g* for 5 min; lanes 3, 7 and 11: 10 000 *g* for 30 min; and lanes 4, 8 and 12: 100 000 *g* for 1 h. The pellets were analyzed by Western blotting using the anti-Envgp70, anti-CAp30, anti-PrP and anti-EF1α antibodies. (**B**) To determine the presence of PrP^{Sc} in the 100K pellet from NIH3T3-22L-MoMuLV cells, the pellets from NIH3T3-N-MoMuLV (negative control, lane 1) and NIH3T3-22L-MoMuLV (lane 2) were treated with PK before immunoblotting with anti-PrP (lanes 3 and 4).

and 12). Analysis with the anti-PrP revealed a very faint PrP signal in the 100K pellet recovered from the NIH3T3-22L supernatant (lane 4). On the other hand, we observed a 20-fold increase in the PrP signal (compare lanes 4 and 8) in the 100K pellet from NIH3T3-22L-MoMuLV supernatant, indicating that MoMuLV infection causes a drastic enhancement of the prion protein release from the infected cells. Identical data were observed with the NIH3T3-N and NIH3T3-N-MoMuLV cell supernatants (data not shown). The observation that most of the PrP signal was associated with the 100K pellet indicates that PrP release in the supernatant is mediated through pelletable structures such as viral particles or, as recently reported, exosomes (Fevrier *et al*, 2004). To determine if exosomes release is enhanced upon MoMuLV infection, we searched for cellular proteins incorporated into exosomes but not found in MoMuLV virions. The translation elongation factor 1α (EF1α) appears to be a good candidate since analyses of purified exosomes by mass spectrometry revealed that EF1α is associated with exosomes (Thery *et al*, 2001; Fevrier *et al*, 2004) but absent from MoMuLV (Cimarelli and Luban, 1999). For this purpose, Western blotting analyses were carried out using antibodies directed against EF1α as an exosome marker. Results show that the production of MoMuLV is associated with a five-fold increase in the EF1α marker (Figure 4A, bottom panel, compare lanes 4 and 8), indicating that MoMuLV infection of NIH3T3-22L cells enhances exosome production.

To determine if PrP^{Sc} is released in the cell culture medium, the 100K pellet from NIH3T3-22L-MoMuLV supernatant was submitted to PK digestion before doing the Western

blotting. As a negative control, we used the 100K pellet from the NIH3T3-N-MoMuLV supernatant. Results presented in Figure 4B revealed the presence of PK-resistant PrP in the 100K pellet of NIH3T3-22L-MoMuLV, thus corresponding to PrP^{Sc} (lane 4), whereas no signal was detected in the control pellet (lane 3).

Fractionation of the 100K pellet on a 10–60% sucrose density gradient (Supplementary Materials and methods) revealed that PrP cofractionates with MoMuLV Gag and Env but also with the EF1α exosome marker at densities 1.1415 and 1.1612 g/cm^3 in the RT peak (Supplementary Figure 3).

Prion proteins are associated with MoMuLV virions and exosomes

Because the anti-PrP antibodies do not specifically detect PrP^{Sc}, virions and exosomes preparations were treated with 3 M guanidine isothiocyanate to enhance PrP^{Sc} immunoreactivity (Taraboulos *et al*, 1990). Detection of PrP^{Sc} on virions and exosomes was carried out by IEM using the anti-PrP antibody followed by protein A gold (15 nm) labeling (Figure 5(a1–a3), see arrows for virions and arrowheads for exosomes). PrP labeling was increased by about two-fold in the presence of guanidine treatment (compare layers a3 and a8), suggesting that additional labelings were specific for PrP^{Sc}. Double immunogold labeling of CAp30/Pr65Gag (PAG 10 nm) and PrP (PAG 15 nm, layers a4–a6) confirmed the presence of PrP^{Sc} on virions. As expected, detection of EF1α revealed that it was exclusively detected on exosomes (layer a7, see white arrowheads for exosomes and white arrows for nonlabeled virions). No labeling was observed using the control (Cont.) antibody (layer a9). We concluded that MoMuLV infection enhances the extracellular release of prion proteins mediated by viral particles and exosomes.

MoMuLV infection enhances the release of prion infectivity

To investigate if MoMuLV infection enhances the release of prion infectivity, coculture experiments were realized (Figure 5B). For this purpose, we cocultured normal NIH3T3-N cells with NIH3T3-N (+/− MoMuLV) and NIH3T3-22L (+/− MoMuLV) as separate cell populations. The first one (NIH3T3-N) was cultured on the bottom surface of a six-well plate (these cells will be named hereafter 'target cells'), while the other cells (NIH3T3-N, NIH3T3-N-MoMuLV, NIH3T3-22L or NIH3T3-22L-MoMuLV) were on four independent inserts containing membranes with 0.4 μm diameter pores, permitting transfer of exosomes and viral particles. Cocultures were realized over 4 days and the target cells were submitted to seven passages (4 weeks). As a control for exchanges between the inserts and the wells, RT activity was monitored on the target cell supernatants 15 days after passage of the cells. As expected, RT activity was detected in wells 2 and 4 (Figure 5B), confirming the passage of virions from the insert to the well and the infection of NIH3T3-N cells. At the end, the target cells were harvested and the presence of PrP^{Sc} was determined using the cell blot assay (Figure 5B; Bosque and Prusiner, 2000). Results revealed that in the absence of PK treatment, all cells express similar levels of PrP. On the contrary, after PK treatment, we observed that only the NIH3T3-N cells cocultured with NIH3T3-22L-MoMuLV cells express PK resistant PrP indicating that the scrapie infectivity was efficiently transmitted under the present experimental conditions. After 16 passages, we failed to detect PK resistant PrP in NIH3T3-N cells cocultured with NIH3T3-22L cells. This observation correlates with the very low level of PrP detected in the NIH3T3-22L supernatant (Figure 4A, lane 4) and confirms that prion infectivity spreading in NIH3T3 cells is not efficient (Vorberg *et al*, 2004a). No prion infectivity transmission was observed when cocultures were realized using inserts containing membranes with 20 nm diameter pores confirming the association of prion infectivity with structures larger than 20 nm (data not shown).

Anti-MoMuLV Envelope antibodies immunoprecipitate prion infectivity

Since PrP^{Sc} is recruited by MoMuLV particles, we analyzed if antibodies directed against the MoMuLV Envelope glycoproteins can immunoprecipitate prion infectivity. For this purpose, magnetic beads were coated with antibodies directed against Envgp70 or the Simian immunodeficiency virus Vpx protein used as an irrelevant antibody. Immunoprecipitations were realized on NIH3T3-22L or NIH3T3-22L-MoMuLV supernatants. RT detection carried out on each immunoprecipitate revealed that condition 4 was positive for RT activity (data not shown) indicating that, as expected, virions were immunoprecipitated. After extensive washings, beads were layered on NIH3T3-N target cells and let over 4 days in contact (Figure 6A). After 4 days, cells were extensively washed to eliminate magnetic beads. Surprisingly, we found that beads from condition 4 were still attached to the cell surface (Figure 6B). This 'sticky' phenotype observed in condition 4 was most probably due to the interaction of the cell surface MoMuLV receptor with free Envgp70 associated with virions bound to the antibody–beads complex. No beads were observed in the other conditions (compare conditions 1–3 with 4).

Target cells were then passaged over 6 weeks until the complete clearance of beads. Then, target cells were analyzed by cell blot assays to detect the presence of PK-resistant PrP (Figure 6C). Results indicate that PrP^{Sc} was only detected in condition 4. No PrP^{Sc} was observed in control experiments, indicating that neither beads nor Vpx were able to immunoprecipitate prion infectivity (Figure 6A–C, conditions 1 and 3). No PrP^{Sc} was detected in condition 2, indicating that prion infectivity was specifically immunoprecipitated by MoMuLV anti-Env in condition 4 (Figure 6C, compare conditions 2 and 4). Thus, these experiments show that MoMuLV anti-Env can immunoprecipitate prion infectivity.

MoMuLV Gag as a key factor for PrP release

The retroviral Gag precursor protein alone is necessary and sufficient to drive the formation and the release of virus-like particles (VLPs). To examine how MoMuLV infection enhances PrP release, we analyzed the respective contribution of GagPol and Env to this process (Figure 7A). For this purpose, NIH3T3-22L cells were transfected with plasmids encoding MoMuLV-GagPol (lane 2), MoMuLV−Env (lane 3), or GagPol + Env (lane 4, see Supplementary Materials and methods). As negative controls non-transfected NIH3T3-22L cells were used (lane 1). After 3 days, the expression of CAp30/Pr65Gag, Envgp70 and PrP was confirmed by Western blotting (Figure 7A, lanes 1–4). No modification of intracellular PrP expression was observed under these different

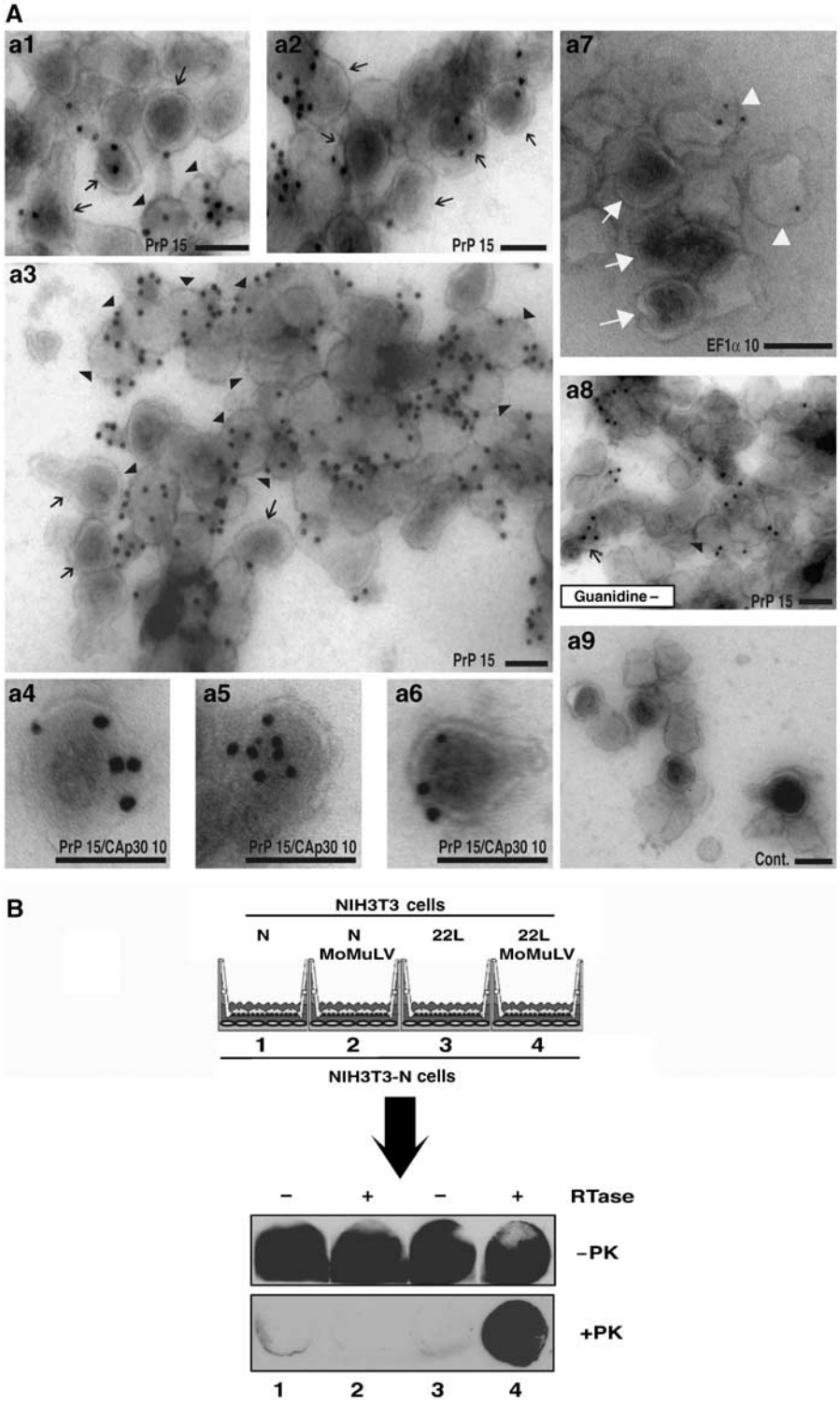

Figure 5 MoMuLV infection strongly enhances the release of prion infectivity. (**A**) PrP^{Sc} released is associated with MoMuLV virions and exosomes. The 100K pellets from NIH3T3-22L-MoMuLV cells were analyzed by IEM for PrP (PAG15), CAp30/Pr65Gag (PAG10) or EF1α (PAG10) after treatment by 3 M guanidium isothiocyanate (5 min). Note the presence of PrP labeling on typical dense viral particle structures (see arrows layers a1–a3) and on light spherical structures corresponding to exosomes (see black arrowheads layers a1 and a3). Single and double IEM for CAp30/Pr65Gag, PrP and EF1α, respectively, on permeabilized virions/exosomes preparation. Layers a4–a6: double IEM for CAp30/Pr65Gag and PrP on MoMuLV virions. Layer a7: EF1α labeling on exosomes (white arrowhead) and not on virions (see white arrows). Layer a9: control (Cont.) irrelevant antibody. Scale bar is 100 nm. (**B**) Coculture assay. The NIH3T3-N cells (target cells) were grown on the bottom surface of a six-well plate and the NIH3T3-N, NIH3T3-N-MoMuLV, NIH3T3-22L and NIH3T3-22L-MoMuLV on the surface of an insert of 0.4 μm pore size. Coculture was carried out over 4 days and target cells were passaged over 4 weeks (seven passages). Transmission of MoMuLV from the insert to the well was controlled by RT assay. Transmission of PrP^{Sc} from the insert cells to the well cells was analyzed by cell blotting in the presence or absence of PK treatment. Immunoblotting was carried out using anti-PrP.

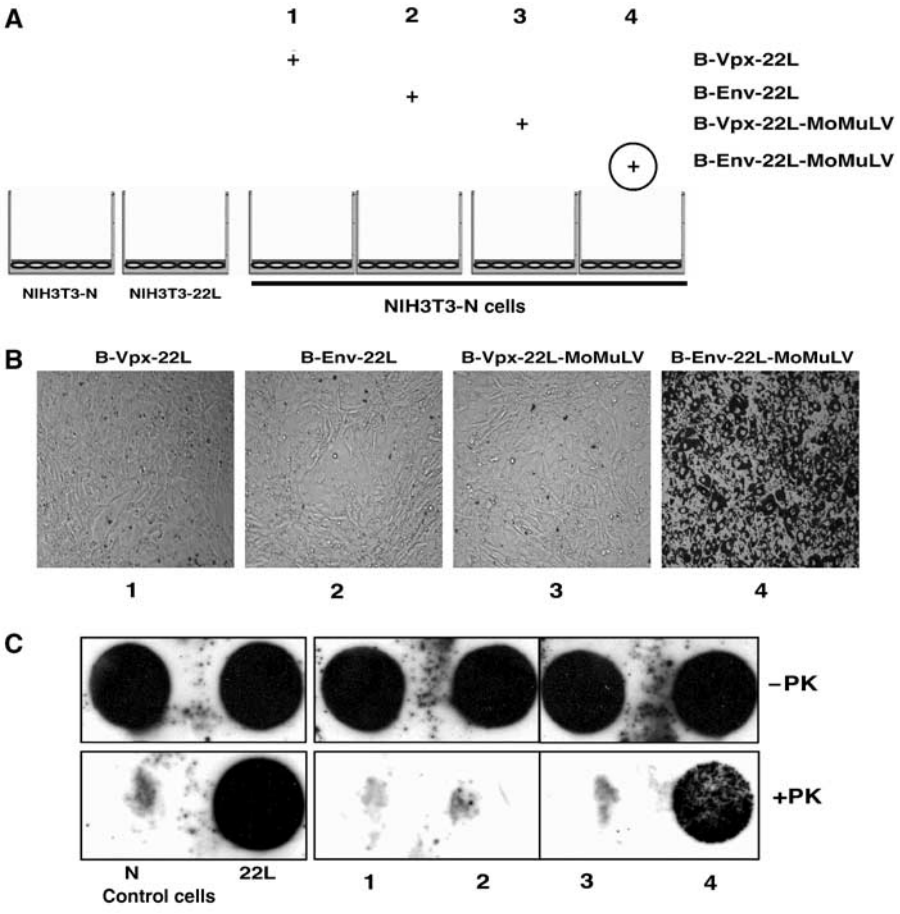

Figure 6 MoMuLV anti-Env antibodies immunoprecipitate prion infectivity. (**A**) Experimental strategy. Magnetic beads coated with MoMuLV anti-Env (conditions 2 and 4) or with SIV anti-Vpx (as irrelevant antibody; conditions 1 and 3) were used for the immunoprecipitation experiments on NIH3T3-22L (conditions 1 and 2) and NIH3T3-22L-MoMuLV (conditions 3 and 4) supernatant. After extensive washing, RT activity was only detected on beads from condition 4 (open circle). Beads were put in contact with NIH3T3-N cells over 4 days. (**B**) Visualization of cell culture by direct light microscopy revealed that beads from condition 4 were still attached to the cell surface after extensive washing. (**C**) Cells were passaged over 6 weeks until the complete loss of beads from the culture. PK-resistant PrP was detected by cell blotting experiment in the presence or absence of PK treatment. As controls of PK treatment, NIH3T3-N and scrapie NIH3T3-22L cells were used (left panel).

conditions (bottom panel). The release of VLPs and virions was monitored by the detection of the RT activity in the cell supernatant (Figure 7B). Results indicated that RT activity was only detected in the supernatant of GagPol and GagPol + Env cotransfected cells. As expected, the level of RT activity was high in cotransfected cells due to the presence of replicative infectious virions (Figure 7B). To determine the role of GagPol and Env, or both in PrP release, the cell supernatant was recovered and centrifuged. The 100K pellets were analyzed by Western blotting using the anti-CAp30 and anti-PrP antibodies (Figure 7C). Results indicate that GagPol production enhanced the PrP release (lane 1), whereas Env expression had no effect (compare lane 2 with lanes 1 and 3). As expected, MoMuLV replication in NIH3T3-22L cells was associated with a strong PrP release (compare lanes 1 and 3), suggesting that the formation and release of VLPs are critical for the enhancement of PrP release.

MoMuLV Gag mutants affecting viral particles release strongly reduce PrP release

To determine if viral particle production is an essential factor involved in the PrP release, we used MoMuLV Gag mutants affecting virus release. The MoMuLV Gag precursor protein comprises four separate domains (Figure 8A), termed matrix (MAp15), p12, capsid (CAp30) and nucleocapsid (NCp10), and all of these regions have been shown to be involved in different steps of viral assembly (Swanstrom and Wills, 1997; Goff, 2001). The p12 domain contains a PPPY motif, which is an important functional domain for MoMuLV assembly and release and is consistent with the notion that it may serve as a Late domain (L-domain) (Figure 8A; Yuan *et al*, 1999, 2000). Deletions of the p12 domain or the DPPPY motif revealed a strong decrease of MoMuLV production (Yuan *et al*, 1999, 2000). Electron microscopical analyses revealed that viral particles were still attached to the cell membrane and connected between them by bridges, confirming that they are defective for virus release (Yuan *et al*, 2000). Similarly, Muriaux *et al* (2004) identified an NC mutant (MoMuLV-ΔNC(16–23); Figure 8A), which affects the release of MoMuLV at a step after trafficking of Gag to the plasma membrane. This prompted us to examine the effect of these three mutants on the release of PrP and compared these with a wild-type (WT) MoMuLV (Figure 8A). For this purpose, NIH3T3-22L cells were transfected with MoMuLV-Δp12 or the

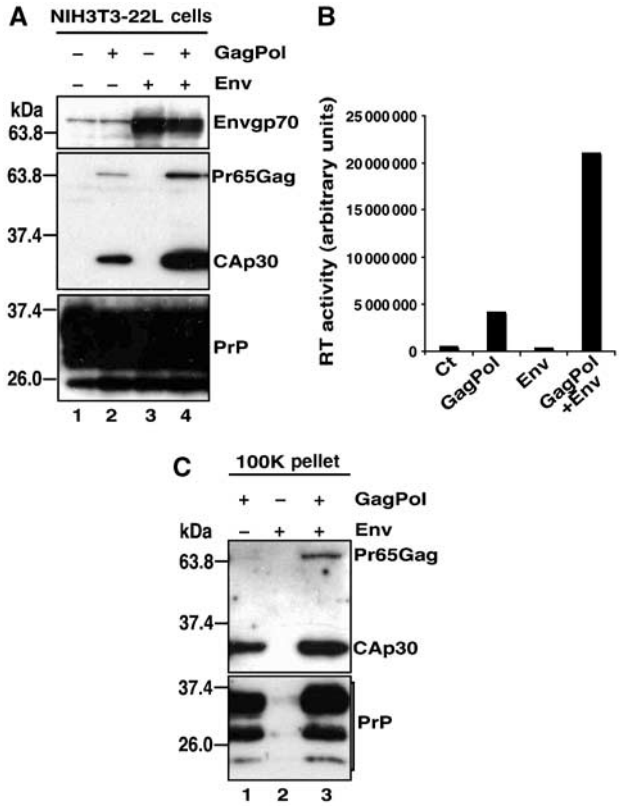

Figure 7 MoMuLV Gag as a key factor involved in PrP release. (**A**) Western blotting analysis of NIH3T3-22L cellular lysate (15 μg) from cells transfected with MoMuLV GagPol, Env and GagPol + Env plasmids and probed with anti-Env, −CAp30 and −PrP antibodies. Lane 1: non-transfected NIH3T3-22L (Ct); lane 2: GagPol; lane 3: Env; and lane 4: GagPol + Env. (**B**) Detection of RT activity. After 3 days transfection, release of VLPs and virions was monitored by measuring RT activity in the cell supernatant. (**C**) GagPol release enhances PrP release. Cell supernatants were centrifuged and the 100K pellet was analyzed by Western blotting using anti-CAP30 and anti-PrP antibodies. Lane 1: 100K pellet GagPol-transfected cells; lane 2: 100K pellet Env-transfected cells; and lane 3: 100K pellet GagPol + Env-transfected cells.

MoMuLV-ΔDPPPY mutant proviral genomes and compared with NIH3T3-22L cells transfected with a WT MoMuLV proviral genome (Figure 8B, lanes 1–3, see Supplementary Materials and methods). After 2 days, the cells were recovered and the expression of CAp30/Gag, PrP and EF1α was monitored by Western blotting using anti-CAp30, anti-PrP and anti-EF1α antibodies (Figure 8B). As expected, the data confirmed an increase of GagΔp12 (lane 2) and GagΔDPPPY (lane 3) proteins compared to the Pr65GagWT (lane 1) correlating with an intracellular accumulation of mutant Gag proteins. No modification of PrP or EF1α expression was observed in the different contexts (bottom panels). To determine if the Δp12 and ΔDPPPY mutants affect MoMuLV release, RT activity in the cell supernatant was determined (Figure 8C). As expected, results confirmed that these mutations affect MoMuLV release. To determine if reduced release of MoMuLV was associated with a decrease of PrP release, virions and exosomes contained in the cell supernatant were pelleted. The 100K pellets were analyzed by Western blotting using anti-CAp30, anti-PrP and the anti-EF1α antibodies (Figure 8D). Results show that reduced MoMuLV release (as judged by the lack of CAp30 signal; compare lane 1 with lanes 2 and 3) is associated with a strong decrease of PrP release (see medium panel). Our data also indicate that reduced release of MoMuLV is also associated with a decrease of exosome release (see EF1α signal, bottom panel). Similar experiments realized with the MoMuLV-ΔNC(16–23) mutant confirmed that PrP and exosome release were also strongly reduced when virus release was inhibited (Figure 8E and F).

Altogether these data indicate that Gag, which drives viral particle formation and release, is a key factor associated with the strong release of PrP and exosome in the extracellular medium.

Discussion

We report here that MoMuLV infection strongly enhances the release of PrP^{C} and PrP^{Sc} proteins and prion infectivity from coinfected cells. Furthermore, we show that prion proteins are released in the supernatant in association with MoMuLV viral particles and confirm their association with exosomes. Our data indicate that viral particles release is a key factor involved in the release of the prion proteins. We propose that retroviruses could be cofactors involved in the spreading of the prion pathological agent.

In line with our findings, it has been proposed that MuLV and endogenous retroelements such as intracisternal A particle (IAP) may be associated with prion infection and suggested that retroviruses could be cofactors involved in prion diseases (Doh-ura *et al*, 1995; Carp *et al*, 1999; Manuelidis, 2003). Indeed, Carp *et al* (1999) showed an interaction between MuLV replication and the scrapie infectious process. Similarly, Chandler (1965) noticed that mice coinfected with Friend tumour's retrovirus and the Chandler scrapie strain display a reduction in extent of tumor induced by the retroviral infection. Analyses of differentially expressed genes in scrapie-infected mouse neuroblastoma cells revealed that IAP Envelope expression was 10-fold increased compared to uninfected cells (Doh-ura *et al*, 1995). Similarly, many reports indicated that IAP Gag proteins and RNAs cosediment with scrapie infectivity (Akowitz *et al*, 1994; Manuelidis *et al*, 1995; Manuelidis, 2003).

Thus, these data suggest that retroelements of exogenous or endogenous origin appear to be closely associated with prion diseases.

Many retroviruses belonging to the MuLV family such as the Cas-Br-E MuLV or the ts1-mutant of MoMuLV severely affect the nervous system (Czub *et al*, 1992; Choe *et al*, 1998). The implications of such retroviruses on prion infectivity release in scrapie-infected mice should be analyzed.

The neuroblastoma N2a cell is a model of choice for prion biology studies since it can be infected with mouse adapted prions and maintain scrapie infectivity over many passages, as assayed in the animal. As previously observed for the GT-1, Mov or Rov cells, we found that scrapie-infected N2a cells release prion infectivity in the culture medium cosedimenting with exosomes but also with endogenous MuLV viral particles (data not shown). The link between the release of infectivity and the presence of virions and exosomes in this cell line is under investigation.

Many reports showed that GPI-anchored proteins such as CD59 or CD55 can be recruited by HIV-1 or MoMuLV virions and that such recruitment is simply due to the presence of

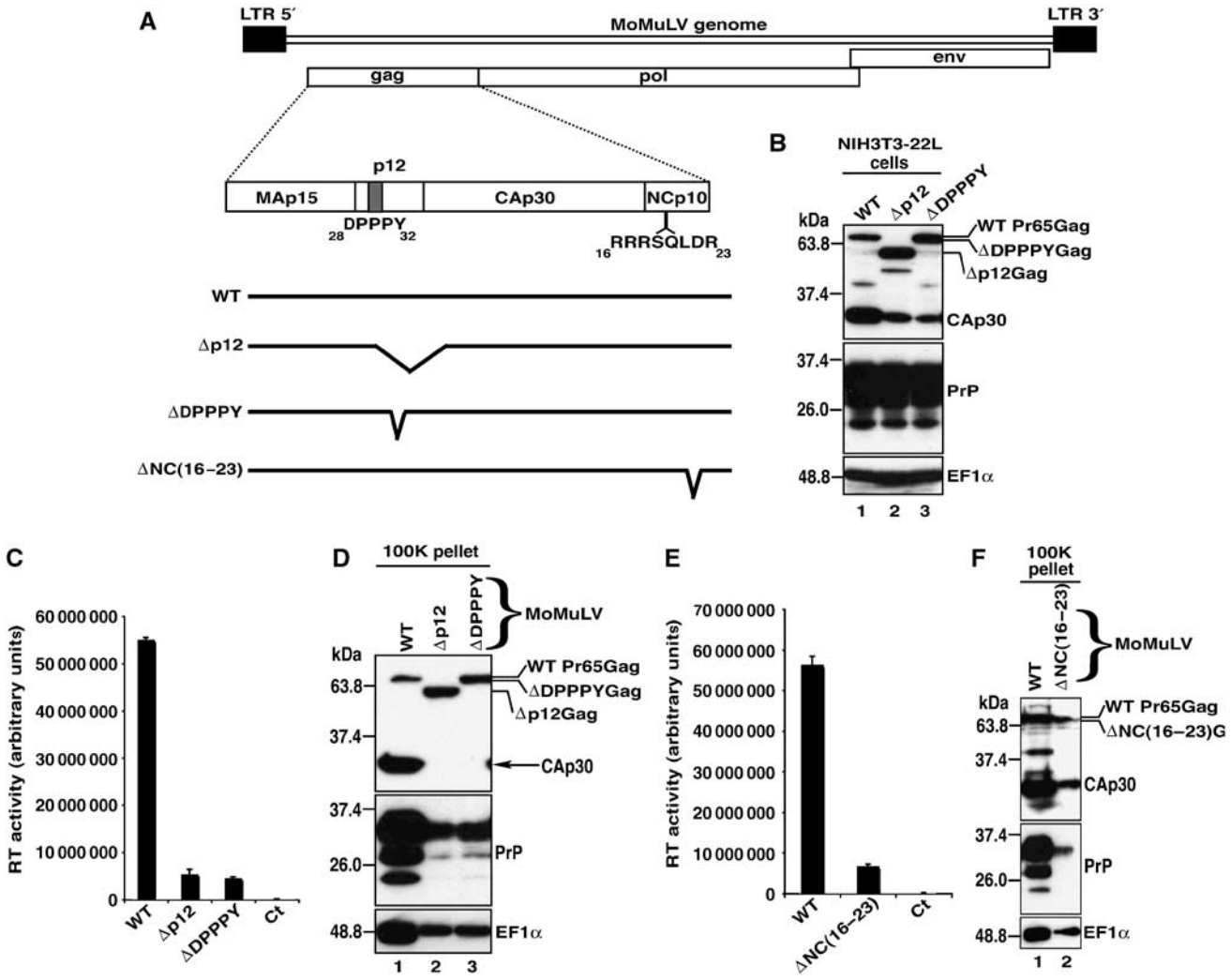

Figure 8 PrP release correlates with MoMuLV release. (**A**) Structure of the MoMuLV genome and of Gag mutants affecting virus release. The *gag*, *pol* and *env* ORF encode, respectively, the Gag precursor composed of the matrix (MAp15), p12, capsid (CAp30) and nucleocapsid (NCp10) domains; the *pol*-encoded enzymes and the Envelope glycoprotein. ORFs are flanked by LTR. WT is the wild-type MoMuLV-Gag, Δp12 is Gag deleted for the p12 domain, ΔDPPPY is deleted for the DPPPY motif (L-domain) and ΔNC(16–23) is a mutant in the nucleocapsid deleted for amino acids 16–23. (**B**) Western blotting analyses of cell lysate (15 μg/lane) from NIH3T3-22L cells transfected with MoMuLV proviral genome DNAs with WT Gag (lane 1) or with mutant Gag Δp12 (lane 2) and ΔDPPPY (lane 3). Blots were probed with anti-CAp30, −PrP and −EF1α antibodies. (**C, E**) Virus production was monitored by measuring the RT activity in the supernatant. Ct corresponds to the supernatant of non-transfected NIH3T3-22L cells. (**D, F**) Release of PrP by MoMuLV-expressing cells. The 100K pellet containing virions and exosomes released were analyzed by Western blotting using anti-CAp30, −PrP and −EF1α antibodies. Lane 1 (D, F): MoMuLV WT; lane 2 (D): MoMuLV Gag-mutant-Δp12; lane 3 (D): mutant-ΔDPPPY; and lane 2 (F): mutant-ΔNC(16–23).

these proteins at the site of assembly and budding of viral particles (Saifuddin *et al*, 1997; Nguyen and Hildreth, 2000; Pickl *et al*, 2001). According to our IEM data, PrP incorporation into the viral particles is likely to be mediated through the viral envelope during assembly and budding steps in DRMs at the plasma membrane and in endosomal compartments. Nevertheless, due to its nucleic acid binding and chaperoning properties (Gabus *et al*, 2001), we cannot exclude that PrP could be recruited to the site of virus assembly through interactions with the viral RNA.

Immunoprecipitation experiments revealed that MoMuLV anti-Env can immunoprecipitate prion infectivity (Figure 6). These findings strongly suggest that prion infectivity is associated with MoMuLV particles or with exosomes containing MoMuLV Envelope glycoprotein. Similarly, it has been found that hepatitis C virus Envelope glycoproteins are recruited by exosomes (Masciopinto *et al*, 2004). Furthermore, analyses of exosome preparations by mass spectrometry revealed that MuLV Gag proteins can be recovered in these vesicular structures (Thery *et al*, 2001). Similarly, Duelli *et al* (2005) proposed that the Mason–Pfizer monkey virus, a member of the type D retrovirus group, was released as an exosome-like particle. Indeed, they reported that during their formation, exosomes could recruit the viral proteins capsid p27 and Envgp70/22. The capacity for exosomes to recruit viral proteins such as Gag and Env could explain why the MoMuLV virions and the EF1α-positive exosomes cofractionate in density and velocity gradients.

Recently, Baron *et al* (2002) indicated that PrP^{Sc} formation is thought to be produced in DRMs. Indeed, Env glycoproteins of HIV-1 and MuLV virions mediate their selective

attachment to the target cell via the receptor and/or coreceptor localized in DRMs (Lu and Silver, 2000; Del Real *et al*, 2002). These data strongly suggest that after viral infection, the prion proteins and the prion infectivity recruited by the viral envelope or the Env-positive exosomes should be inserted directly into the DRMs, at the plasma membrane and/or in intracellular compartments.

Here we show for the first time that retroviral infection strongly enhances the release of prion proteins and scrapie infectivity. Furthermore, our investigations showed that the retroviral Gag are important in this mechanism since mutants of Gag affecting viral particles release do not cause an enhancement of prion protein release. In the course of our investigations, we also found that Gag release stimulates the exosome liberation. Recently, several reports indicated that Gag hijacks the budding machinery of MVBs (Morita and Sundquist, 2004). The sorting and budding machinery of MVB is composed of multiprotein complexes termed ESCRT-I to -III (for Endosomal Sorting Complex Required for Transport). In addition, many other cellular proteins are associated with these complexes for the progress of the protein sorting and vesicle formation. Many components belonging to the ESCRT complexes interact with Gag directing the budding and the release of retroviral particles in MVBs (Morita and Sundquist, 2004). Yeast two-hybrid studies realized on MoMuLV Gag revealed that it interacts with ubiquitin ligases of the Nedd4 family through the PPPY motif, TSG101 (ESCRT-I) through the PSAPS motif or with Alix/Aip1 through the LYPAL motif and that such partners of Gag could play a role in the MoMuLV release (Segura-Morales *et al*, 2005). Garrus *et al* (2001) found that expression of a human mutant of the Vps4 ATPase strongly reduced the release of MoMuLV in human cell culture. Similarly, Goila-Gaur *et al* (2003) observed that expression of a truncated form of human TSG101 (TSG-3′) inhibits the release of MoMuLV. To better characterize the cellular mechanisms by which MoMuLV production enhances the release of prions and exosomes, the expression of such cellular dominant-negative proteins and the use of siRNA directed against these cellular partners should be investigated.

Similarly, we found that infection of a lymphoblastoid cell line by the HIV-1 induces a strong release of human PrP^C in the supernatant (data not shown). This result indicates that release of prion protein induced after retroviral infection is not restricted to MoMuLV, suggesting that it could correspond to a widely virally induced process. Indeed, we found that transient expression of the vesicular stomatitis virus glycoprotein (VSVg) in NIH3T3-N cells strongly enhances the release of PrP^C in the cell supernatant (data not shown). Experiments are currently initiated to determine if expression of VSVg protein in scrapie-infected cells can lead to the increase of prion infectivity in the cell supernatant. These new findings highlight the possible implications of enveloped viruses on prion proteins and scrapie infectivity release by infected cells.

The small ruminant lentiviruses Maedi Visna virus (MVV) and the caprine arthritis encephalitis virus (CAEV) largely infect sheep flocks and goat herds and are endemic in most, if not all, European populations of small ruminants (Peterhans *et al*, 2004; Ligios *et al*, 2005). How Scrapie spreads in these populations is still enigmatic. In agreement with our findings, the small ruminant lentiviruses MVV and CAEV might be viral cofactors involved in prion propagation. Ligios *et al* (2005) report the presence of PrP^{Sc} in mammary glands of sheep suffering from scrapie and mastitis and found a good correlation with MVV seropositivity. They proposed that common viral infections (such as MVV) of small ruminants may enhance the spread of prions.

Taken together, we found that PrP^C, PrP^{Sc} and prion infectivity release is drastically enhanced upon retroviral infection. Thus, we propose that retroviruses could be critical cofactors involved in the spread of the pathological prion agent.

Materials and methods

Plasmids and cells

pMLVH2neo (Housset *et al*, 1993), pNCS (Yuan *et al*, 1999), pRR88 (Muriaux *et al*, 2004) and pNL4-3 constructs (Adachi *et al*, 1986) are infectious molecular clone of MoMuLV and HIV-1, respectively. pNCSΔp12 and pNCSΔDPPPY are MoMuLV Gag mutants deleted for the p12 domain and the DPPPY motif contained in p12 of the Gag protein (Yuan *et al*, 1999, 2000). pRR88ΔNC(16–23) is an MoMuLV Gag mutant deleted for the amino acids 16–23 of the nucleocapsid domain (Muriaux *et al*, 2004). pNCS, pNCSΔp12 and pNCSΔDPPPY were kindly provided by S Goff and pRR88 and pRR88ΔNC(16–23) by A Rein. All MoMuLV Gag mutants have been realized in a full-length MoMuLV proviral genome encoding functional Pol and Env proteins. pGPP plasmid encoding MoMuLV GagPol under the long terminal repeat (LTR) promoter was a gift from A Telesnitisky (Shin *et al*, 2000) and MoMuLV Envelope expression plasmid FBMoSALF was from FL Cosset (Cosset *et al*, 1995).

NIH3T3i cell line corresponds to NIH3T3 cells transfected with pMLVH2neo and selected with G418 antibiotic. NIH3T3-N (normal) and NIH3T3-22L (scrapie-infected) cells were kindly provided by S Priola (Vorberg *et al*, 2004b). These cells have been transfected with pMLVH2neo DNA to establish NIH3T3-N-MoMuLV and NIH3T3-22L-MoMuLV cell lines, which constitutively produce MoMuLV virions. 293T human embryonic kidney cell line was obtained from the Généthon (Evry, France). Cells were cultured in DMEM medium (Invitrogen) supplemented with 10% FCS, L-glutamine and penicillin/streptomycin.

Antibodies

Monoclonal anti-PrP antibodies SAF-37, SAF-32 (recognizing the entire octa-repeat region), SAF83 (conformational epitope located within residues 126–164) were obtained from J Grassi (CEA Saclay, SPI/CEA). HIV-1 antibodies αEnv-SUgp120 (ref. 2640, IgG1b12) was obtained from the NIH (AIDS research and reference reagent program). HIV-1 αCAp24 antibody was from Biomerieux (Lyon, France), α-cyclophylin A was a gift from P Gallay. Biotinylated cholera toxin fragment (BCTx) and streptavidin–peroxidase complex were from Sigma. Goat α-Env-gp70 RLV and α-CAp30 RLV were from Quality Biotech. Rabbit α-Env-gp70 (DJ-39462) and α-CAp30 (DJ-39461) antibodies specific of MoMuLV were kindly provided by RJ Gorelick (AIDS Vaccine program, SAIC-Frederick). α-Lamp1 (ref. sc-19992) and the α-Rab11 (ref. sc-10767) were purchased from Santa Cruz. SIV rabbit anti-Vpx was provided by A Cimarelli. Rabbit, mouse and goat secondary antibodies were purchased from DAKO.

Virus production and purification

Cell culture supernatants containing viral particles were harvested, clarified by centrifugation and filtrated through a 0.45 μm pore membrane (Millipore, MA). MoMuLV virions were concentrated through a 20% sucrose cushion at 100 000 *g* in SW28 or SW41 rotor (Beckman) and viral pellets were directly analyzed in sample buffer by Western blotting or separated through a 6–18% optiprep gradient (Dettenhofer and Yu, 1999).

Differential centrifugations

Cell culture medium from NIH3T3-N, NIH3T3-N-MoMuLV, NIH3T3-22L and NIH3T3-22L-MoMuLV cells were centrifuged 5 min at 3000 *g* and 5 min at 4500 *g* to remove cells in suspension, ultracentrifuged 30 min at 10 000 *g* to remove cellular debris and

finally at 100 000 *g* for 1 h to pellet virions and exosomes. The resulting pellets were directly resuspended in sample buffer and analyzed by Western blotting or submitted to PK treatment before immunoblotting to detect the PrP^{Sc}.

Optiprep gradient centrifugation

MoMuLV virions produced by NIH-3T3-infected (NIH3T3i) cells and HIV-1 virions from supernatant of HIV-1-transfected 293T-PrP cells were recovered, filtered and pelleted through a 20% sucrose cushion (2 h at 122 000 *g* in a SW28 rotor). The viral pellet was resuspended in PBS containing a protease inhibitor cocktail (Complete Mini, Roche) during 1 h at 4°C, layered onto a 6–18% optiprep (Nycomed Gibco BRL) velocity gradient and centrifuged for 1.5 h at 176 000 *g* in an SW41 Ti rotor. Fractions (700 µl) were collected from the top, treated with sample buffer (50 mM Tris–HCl (pH 6.8), 5% 2-mercaptoethanol, 10% glycerol, 1% sodium dodecylsulfate (SDS), 0.001% bromophenol blue) and 30 µl of each fraction was analyzed by Western blotting using, for MoMuLV the rabbit anti-CAp30 and anti-PrP (SAF32) antibodies, and the BCTx for the detection of the GM1 raft marker or for HIV-1 the anti-CAp24, the anti-PrP (SAF37) and the anti-CypA antibodies.

Subcellular fractionation

Separation of different membrane compartments was achieved as described previously (Kolesnikova *et al*, 2004) with some modifications. Briefly, NIH3T3i cells were washed, scraped in cold PBS and homogenized in 1 ml of buffer 1 (10 mM Hepes (pH7 .4), 0.25 M sucrose, 1.5 mM $MgCl_2$) for 20 min at 4°C. Cells were disrupted by passaging 12 times through a 25 G needle. Nuclei were pelleted twice at 800 *g* for 10 min at 4°C. The supernatant was collected and centrifuged again to remove any residual nuclei. Subcellular fractionation was performed in three-step iodixanol gradients. Briefly, one-third of the postnuclear supernatant was mixed with 60% optiprep and buffer 1 to generate 10, 20 and 30% iodixanol solutions. Equal volumes of these three solutions were layered in SW60Ti centrifuge tubes and samples were centrifuged at 260 000 *g* for 3 h at 4°C in an SW60Ti rotor. Fractions (150 µl) were collected from the top and proteins were separated by SDS–PAGE and transferred onto a PVDF membrane. Membranes were probed with antibodies directed against CAp30, Envgp70, Lamp1, Rab11 and PrP.

Prion infectivity immunoprecipitation with MoMuLV anti-Env

Immunoprecipitation of prion infectivity by the MoMuLV anti-Env was assessed using Dynabeads G-protein (Dynal) coated with the rabbit α-Env-gp70 (DJ-39462) and the rabbit α-Vpx as irrelevant antibody. Coating was realized according to the manufacturer's conditions. Immunoprecipitations were carried out on filtrated NIH3T3-22L and NIH3T3-22L-MoMuLV supernatants. Beads were extensively washed with PBS before RT activity detection and for the contact experiment with NIH3T3-N cells. After 4 days of contact, cells were extensively washed with PBS and culture medium. At this step, only the beads-Env-22L-MoMuLV were still attached to the cell surface. Cells were then passaged over 6 weeks until the complete clearing of beads from the culture. Detection of PK-resistant PrP was carried out by cell blot assay.

Cocultures

For the coculture experiments, the NIH3T3-N target cells were plated at 2.5×10^5 cells density onto the growth surface of a six-well plate. For insert cocultures, NIH3T3-22L-MoMuLV cells and, as controls the NIH3T3-N, NIH3T3-N-MoMuLV and NIH3T3-22L cells were plated at 2.5×10^5 cells onto the growth surface of a 4.2 cm^2 membrane with high-density pores of 0.4 µm diameter (Becton Dickenson). Contact between cells is realized through the culture medium only. Cocultures were realized over 4 days and target cells were passaged over 4 weeks (seven passages).

Cell blot assay

To detect the PK-resistant PrP, cells were cultured on glass coverslips and transferred onto nitrocellulose membrane or blotted onto nitrocellulose membrane using a home-made cell blot apparatus. Detection of PrP^{Sc} was realized as described previously (Bosque and Prusiner, 2000).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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